# BONES, MUSCLES AND VITAMIN C

## I. THE EFFECT OF A PARTIAL DEFICIENCY OF VITAMIN C ON THE REPAIR OF BONE AND MUSCLE IN GUINEA-PIGS*

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### INTRODUCTION

The observations described in this paper, some of which have been mentioned in earlier communications (Kodicek & Murray, 1943; Murray & Kodicek, 1946), were made in the course of an investigation, which had negative results, of the alleged reopening of old healed fractures in prolonged vitamin C insufficiency. We found no evidence of reopening of healed fractures under these conditions, but we made certain other observations, the first three of which are the subjects of the present paper. These observations concerned (1) the repair of the fractures; (2) the appearance of a contracture at the knee joints; (3) the failure of injured muscles to regenerate and their replacement by hyperplastic connective tissue; (4) the development of subperiosteal thickenings of the diaphyses of certain bones and other anatomical changes. These last are treated in the second and third papers of this series.

### EXPERIMENTS, MATERIAL, METHODS

The animals used were guinea-pigs. The weights of the vast majority, at the beginning of the experiment, were between 200 and 400 g. The basal diet was in the earlier experiments 720 g. oatmeal, 180 g. bran, 20 g. salt mixture, 100 g. dried yeast; later, the bran and yeast were changed to 160 and 80 g. respectively. To the basal diet we added ascorbic acid and radiostoleum. Carotene or carrots were given, as supplements of vitamin A, to the diets of those deprived of vitamin D. The supplements given in the different experiments are summarized in Table 1. In the diets which were partially deficient in vitamin C, our aim was to reduce the intake of the vitamin to as low a level as we could, while preventing obvious scurvy and death; in some experiments we gave 0·5 mg., in others 1 mg. ascorbic acid daily. The animals were weighed on alternate days, or sometimes daily.

The fractures were inflicted under ether anaesthesia with forceps resembling those used by Hertz (1936). Following Hertz, the fibula was chosen for fracturing because the tibia made an effective splint and there was usually good apposition of the ends. No open operation was required, but the method undoubtedly involved injury to the soft tissues by squeezing.

* Parts II and III will appear in subsequent issues of the *Journal*.

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In all experiments, except Exp. 2, a radiograph was taken to confirm that a fracture had been made, and further radiographs were taken later at intervals of from a fortnight to a month.

The animals were killed with chloroform. The operated limb, and often both posterior limbs and other parts, were fixed in Heidenhain's 'Susa' solution. Specimens were at first embedded by Apathy's celloidin-paraffin method, but later in paraffin; the latter method provided more complete series of sections. Sections were stained with haematoxylin and eosin, Heidenhain's 'Azan', Wilder's silver method, van Gieson, Giemsa, and other methods. When animals died before being killed, similar material was collected and preserved in either 10 % formol or Susa.

Table 1 summarizes the nine experiments, showing how the diets were varied, the numbers of animals, the dates of the operation when one was performed, and the dates of death. 'Experiment 1' is really a list of all the dietetically normal animals, including some of those also listed as group 1 in Exps. 2–6, in which a fractured fibula was studied histologically.

## PARTIAL DEFICIENCY OF VITAMIN C AND DEPRIVATION OF VITAMIN D, AND THE REPAIR OF FRACTURES

The diets deficient in vitamin D, in which the balance of salts was not altered, produced neither rickets nor any delay in callus formation at the fractures, nor indeed any other effect, whether alone or combined with a partial deficiency of vitamin C.

The effects of partial and complete vitamin C deficiencies on early stages in the repair of bone have been studied by Bourne (1942, 1943, 1944) and others (Hanke, 1935; Hertz, 1936; Israel, 1926; Israel & Fränkel, 1926; Jeney & Korpássy, 1934; Lexer, 1939; Roegholt, 1931–2; Schilowzew, 1928; Watanabe, 1924; Wolbach & Howe, 1925, 1926), but the influence of prolonged partial deficiencies, which we studied in Exps. 2–6 is less well known.

The radiographic findings and the results of the microscopical investigation will be discussed separately.

### *Radiographs*

In Exp. 5, radiographs were taken of the operated limbs immediately after the operation and at 17, 32, 59, and 94 days later, and in Exp. 6 immediately after the fracture and at 19, 50 and about 80 days after the fracture. In group 1 (fully supplemented diets) of both experiments, the radiographs (Pl. 1, figs. 1, 3) taken at 17 or 19 days showed a callus with a narrow uncalcified line across it, while in those taken at 32 or 50 days no such line was visible and the callus was so reduced as to make the exact position of the fracture difficult to recognize. In groups 3 (partially deficient in vitamin C) and 4 (partially deficient in vitamin C and deprived of vitamin D), the amount of callus formed (at least at the site of the fracture) at 17 or 19 days was much less (Pl. 1, figs. 2, 4). In addition, the resorption of bone from the broken ends was usually greater than in the control animals (group 1) as shown by the greater width of the clear line between them. Even at 32 or 50 days, traces of the gap, or even a clear area right across the bone, were often visible (Pl. 1, fig. 2).

TABLE 1. *A summary of the dietary vitamin supplements given, of the times at which supplements were changed, of the times of operation, and of dates of death. Exp.* 1 *includes all animals, on fully supplemented diets throughout, whose fractured fibulae were sectioned*

| Exp. | Group | No. of animals | Days of experiment | Vitamin C supplement | Vitamin D supplement | Vitamin A supplement | Day of operation | Days of experiment on which animals died or were killed (in brackets: number of animals) |
|---|---|---|---|---|---|---|---|---|
| 1 | — | 34 | — | Fully supplemented | | | 1 | 1–7 (1 each), 9 (2), 10–14 (1 each), 15 (4) 16 (5), 63 (1), 81 (2), 83 (2), 84 (2), 85 (1), 94 (2), 101 (1) |
| 2 | 1 | 10 | 1–14 | Cabbage | 6 drops radiostoleum weekly | | 1 | 25 (1), 30 (1), 33 (1), 42 (1), 53 (1), 60 (1), 65 (1), 85 (3) |
| | | | 15–25 | None | ,, | ,, | | |
| | | | 26–end | 0·5 mg. A.A. daily* | ,, | ,, | | |
| | 2 | 4 | 1–end | Cabbage | 6 drops radiostoleum weekly | | 1 | 85 (4) |
| 3 | 1 | 7 | 1–end | 10 mg. A.A. daily | 6 drops radiostoleum weekly | | 1 | 16 (3), 102 (4) |
| | 2 | 8 | 1–end | 10 mg. A.A. daily | None | 8 g. carrots weekly | 1 | 16 (3), 18 (1), 54 (1), 102 (3) |
| | 3 | 4 | 1–15 | 10 mg. A.A. daily | 6 drops radiostoleum weekly | | 1 | 42 (1), 102 (3) |
| | | | 16–32 | 0·5 mg. A.A. daily | ,, | ,, | | |
| | | | 33–60 | 1·0 mg. A.A. daily | ,, | ,, | | |
| | | | 61–end | 0·5 mg. A.A. daily | ,, | ,, | | |
| | 4 | 12 | 1–15 | 10 mg. A.A. daily | None | 8 g. carrots weekly | 1 | 31 (1), 32 (1), 42 (1), 69 (1), 88 (1), 102 (2), 103 (5) |
| | | | 16–32 | 0·5 mg. A.A. daily | ,, | ,, | | |
| | | | 33–60 | 1·0 mg. A.A. daily | ,, | ,, | | |
| | | | 61–end | 0·5 mg. A.A. daily | ,, | ,, | | |
| 4 | 2 | 4 | 1–end | 10 mg. A.A. daily | None | 8 g. carrots weekly | 1 | 15 (1), 81 (3) |
| | 4 | 4 | 1–14 | 10 mg. A.A. daily | ,, | ,, | 1 | 81 (4) |
| | | | 15–37 | 1·0 mg. A.A. daily | ,, | ,, | | |
| | | | 38–end | 0·5 mg. A.A. daily | ,, | ,, | | |
| 5 | 1 | 4 | 1–end | 10 mg. A.A. daily | 6 drops radiostoleum weekly | | 27 | 121 (4) |
| | 2 | 4 | 1–end | 10 mg. A.A. daily | None | 10 drops carotene twice weekly | 27 | 52 (1), 91 (1), 107 (1), 121 (1) |
| | 3 | 5 | 1–7 | 1·0 mg. A.A. daily | 6 drops radiostoleum weekly | | 27 | 63 (1), 67 (1), 113 (2), 121 (1) |
| | | | 8–18 | None | ,, | ,, | | |
| | | | 19–end | 0·5 mg. A.A. daily | ,, | ,, | | |
| | 4 | 5 | 1– 7 | 1 mg. A.A. daily | None | 10 drops carotene twice weekly | 27 | 45 (1), 47 (1), 63 (1), 93 (1), 120 (1) |
| | | | 8–18 | None | ,, | ,, ,, | | |
| | | | 19–end | 0·5 mg. A.A. daily | ,, | ,, ,, | | |

| Exp. | Group | No. of animals | Days of experiment | Vitamin C supplement | Vitamin D supplement | Vitamin A supplement | Day of operation | Days of experiment on which animals died or were killed (in brackets: numbers of animals) |
|---|---|---|---|---|---|---|---|---|
| | 5 | 2 | 1– 7 | 1 mg. A.A. daily | | 6 drops radiostoleum weekly | None | 62 (1), 121 (1) |
| | | | 8–18 | None | | ,, ,, | | |
| | | | 19–end | 0·5 mg. A.A. daily | | ,, ,, | | |
| | 6 | 2 | 1– 7 | 1·0 mg. A.A. daily | None | 10 drops carotene twice weekly | None | 61 (1), 63 (1) |
| | | | 8–18 | None | ,, | ,, ,, | | |
| | | | 19–end | 0·5 mg. A.A. daily | ,, | ,, ,, | | |
| 6 | 1 | 4 | 1–end | 10 mg. A.A. daily | | 6 drops radiostoleum weekly | 24 | 98 (1), 105 (2), 109 (1) |
| | 2 | 4 | 1–end | 10 mg. A.A. daily | None | 10 drops carotene twice weekly | 24 | 47 (1), 79 (1), 85 (1), 99 (1) |
| | 3 | 4 | 1– 7 | 1·0 mg. A.A. daily | | 6 drops radiostoleum weekly | 24 | 49 (1), 51 (1), 95 (1), 109 (1) |
| | | | 8–16 | None | | ,, ,, | | |
| | | | 17–end | 0·5 mg. A.A. daily | | ,, ,, | | |
| | 4 | 5 | 1– 7 | 1·0 mg. A.A. daily | None | 10 drops carotene twice weekly | 24 | 30 (2), 51 (1), 65 (1), 85 (1) |
| | | | 8–16 | None | ,, | ,, ,, | | |
| | | | 17–end | 0·5 mg. A.A. daily | ,, | ,, ,, | | |
| | 5 | 2 | 1–7 | 1·0 mg. A.A. daily | | 6 drops radiostoleum weekly | None | 95 (1), 109 (1) |
| | | | 8–16 | None | | ,, ,, | | |
| | | | 17–end | 0·5 mg. A.A. daily | | ,, ,, | | |
| | 6 | 2 | 1– 7 | 1·0 mg. A.A. daily | None | 10 drops carotene twice weekly | None | 72 (1), 109 (1) |
| | | | 8–16 | None | ,, | ,, ,, | | |
| | | | 17–end | 0·5 mg. A.A. daily | ,, | ,, ,, | | |
| 7 | 1 | 12 | 1–10 | None | | 6 drops radiostoleum weekly | None | 54 (1), 62 (1), 64 (2), 66 (8) |
| | | | 11–end | 0·5 mg. A.A. daily | | ,, ,, | | |
| | 2 | 8 | 1–10 | None | | 6 drops radiostoleum weekly | None | 72 (1), 86 (1), 107 (6) |
| | | | 11–66 | 0·5 mg. A.A. daily | | ,, ,, | | |
| | | | 67–end | 10 mg. A.A. + cabbage daily | | ,, ,, | | |
| 8 | 1 | 6 | 1–end | None | | 6 drops radiostoleum weekly | None | 16 (1), 17 (5) |
| | 2 | 8 | 1–16 | None | | 6 drops radiostoleum weekly | None | 66 (8) |
| | | | 17–end | 15 g. cabbage daily | | ,, ,, | None | |
| 9 | — | 16 | 1– 8 | 1·0 mg. A.A. daily | | 6 drops radiostoleum weekly | 27 | 29 (3), 31 (3), 34 (3), 37 (2), 40 (2), 42 (1), 44 (2) |
| | | | 9–18 | None | | ,, ,, | | |
| | | | 19–end | 0·5 mg. A.A. daily | | ,, ,, | | |

* A.A. = Ascorbic acid

*Microscopical investigation*

Sections confirmed these differences between the dietetically normal and partially vitamin C-deficient animals.

*Normal animals*

It is not necessary to give a full description of normal fracture repair, but certain points must be mentioned for comparison with events in the animals on experimental diets. The formation of new bone did not begin at the fracture itself, but at some distance from it in the swollen cambial layer of the periosteum; later, it gradually approached the fracture site. At about the end of the first week after the operation, cartilage might begin to appear in the densely cellular tissue in which the broken ends were now embedded. One of the two animals (Pl. 2, fig. 5) killed at 9 days showed a considerable quantity of cartilage forming in the fracture region, while the subperiosteal bone had reached the fracture and trabeculae growing out from the proximal and distal fragments, each towards the other, tended to link up around it. In the other 9-day specimen (Pl. 2, fig. 6) killed at this time, neither bone nor cartilage had yet appeared at the fracture site, though subperiosteal bone was actively developing at a short distance from the broken ends. In all the animals killed at from 10 to 14 days (Pl. 2, fig. 7), bone formation had spread to the fracture, and trabeculae had formed across it, making a sort of basket of trabeculae connecting the proximal and distal subperiosteal tissues. At first no bone formed centrally, between the broken ends themselves, but centrally situated trabeculae were present after 14 days. Cartilage was present in most cases. It tended to form in the peripheral parts of the cellular tissue which joined the fragments before the appearance of bone, but it was later often found in the central region between the ends. It was finally eroded and replaced by bone. In succeeding days (Pl. 2, fig. 8) the trabeculae of the callus became stronger and more numerous, and the cartilage, except for vestiges enclosed in bony trabeculae, disappeared.

The condition of the fractured region was investigated in eleven animals killed at times ranging from 81 to 101 days after the operation (Pl. 3, fig. 9). In all these the callus was recognizable in longitudinal sections from its disorderly structure. It differed from the callus of 2–3 weeks after fracture in two important respects. First, further deposition of bone on the trabeculae, and doubtless the formation of new trabeculae, had transformed the lightly built early callus into massive bone in which the solid substance predominated over the vessel-containing spaces, the opposite of the earlier condition. Secondly, the marked local expansion of the fibula, made by the early callus, had disappeared, and sections showed that the projecting part had been removed by osteoclastic resorption from the surface.

*Partially vitamin C-deficient animals*

Fracture repair in animals placed on the experimental diets before the infliction of the fracture was studied in Exps. 5, 6 and 9. The material consisted of longitudinal sections through the fractured region in animals killed at the following number of days after operation (the numbers in brackets give the numbers of days between

beginning the experimental diets and death): 14 (40), 16 (42), 18 (45), 20 (47), 27 (51), 36 (63, two animals), 40 (67), 61 (85), 86 (113), 93 (120).

The tendency, seen in the radiographs, of many animals on the experimental diets to show more resorption at the broken ends than the controls did, was confirmed by the animal killed at 14 days (Pl. 3, fig. 10) in which there was a long gap between the fragments, filled by a cellular and fibrous tissue. No repair had yet begun at the fracture itself. In none of the animals on the normal diet and killed after 9 days was the repair process still in so early a stage; in all, cartilage had formed and bony trabeculae were crossing the gap.

In the experimental animals which died or were killed at 16, 18 (Pl. 3, fig. 11) and 20 days after the operation, a few trabeculae had joined across the gap, making a feeble connexion, but there was no cartilage, and the repair accomplished was very much less than in the normal animals. There was, however, great variation in the effect of the diet, and the animal which died at 18 days showed repair indistinguishable from that expected in an animal on a normal diet.

In most of the material from animals kept for longer periods, the new subperiosteal bone which, as in normal animals, first developed at some distance from the site of the fracture, had increased enormously and had formed a great thickening often extending along the whole length of the diaphysis. These diaphyseal thickenings, which will be described in detail in the second paper of this series, were trabecular structures resembling lightly built calluses of great extent. In the two animals at 36 days after the operation, and in that at 61 (Pl. 3, fig. 12), the two pieces of the fibula were connected by the new bone extending over them like an immense callus. In the one which died at 93 days a pad of cartilage remained, still unresorbed, between the ends (Pl. 3, fig. 13). In those at 40 and 86 days, in which no large thickening had developed, they were weakly joined by small calluses developed between and around their ends. The chief fact of interest is that, whether the region of the fracture was, or was not, enclosed in a large mass of new bone, the repair of the fracture was never completed as it was in the controls, for the callus, large or small, always remained of light trabecular construction and was never consolidated as it was in the animals on properly supplemented diets. Compare Pl. 3, figs. 12 and 13, with fig. 9.

Pl. 4, fig. 14, shows the fractured fibula of an animal of Exp. 4, group 4, in which the partially deficient diet did not begin until 2 weeks after the operation, and in which there was no period of total deficiency. Nevertheless, the callus, which must have been formed when the partial deficiency began, failed to consolidate. At least one other case behaved similarly.

## STIFFNESS OF THE KNEES

The most constantly observed change at the joints was a development of stiffness at the knees. This occurred in the great majority of partially vitamin C-deficient animals. Thus, in Exp. 9, in which the condition was most completely recorded, some degree of stiffness was found in both legs of all sixteen animals. It was never seen in guinea-pigs adequately supplied with vitamin C, and we did not notice it in other joints. Usually the leg was fixed in flexion, but sometimes in extension; in

severe cases we were unable to move the joint without using force which we feared would cause a fracture, while in others the knee could be moved with less violence but with evident pain. The condition has been described by Meyer & McCormick (1928), who give a photograph. Stiffness did not occur in animals provided with vitamin C but deprived of vitamin D.

The stiffening of the knees seems to be one of the earliest signs of a partial deficiency of the vitamin. In Exps. 5, 6 and 9 it was already present on the day of the operation, that is on the 24th and 27th days of the experimental diet, which had included a period of total deficiency of vitamin C followed by partial deficiency. Unfortunately, we do not know whether, in the present experiments, the stiffness appeared during the period of total deficiency or not, but in more recent experiments by one of us (E. K.) it did not appear during total deprivation lasting 18 days. That total deficiency is not necessary for the appearance of stiffness is shown by Exps. 3 and 4, in which there was no total deprivation, and in which stiffness appeared in at least thirteen out of thirty-two legs examined.

Stiffness is also not a result of the operation, for in Exps. 3–6, among thirty legs operated, stiffness developed in nineteen but not in the other eleven, while of twenty-six unoperated legs, seventeen showed stiffness.

There was a distinct correlation between the right and left sides of the same animal: in the experiments just mentioned, fifteen showed stiffness of both legs, eight of neither, and only three showed it in one leg and not in the other. Stiffness thus depends rather on the diet than on the injury.

Meyer & McCormick (1928) attributed stiffness to changes which they observed in the spinal cord. We made no study of the nervous system, but found that the knees remained stiff after death, and in Exp. 9 we studied the effect of removing the muscle crossing the knee joint. This lessened the stiffness in ten cases but left it unaffected in nine. We are thus disinclined to regard the condition as wholly of central nervous origin. In the next section we shall describe a swelling of the soft tissues of the shank, with degeneration of the muscles and their replacement by a mass of hyperplastic connective tissue. This condition would certainly help to prevent active movement of the limbs and in extreme cases the connective tissue, by packing around the joint and thickening its capsule, may have lessened freedom of movement. The articular surfaces themselves nearly always appeared normal. An attempt to correlate stiffness of the knees with pathological changes in the musculature has an indefinite result. In the animals more severely affected by the partial deficiency, there were such characteristic scorbutic changes as haemorrhages about the knee and even into the joint cavity, and this doubtless contributed to the stiffness.

We incline to the view that stiffness is caused locally rather than in the central nervous system, and that a number of histological changes contribute, these including degenerative and oedematous changes in the muscles, and the formation of hyperplastic connective tissue at and around the joint.

## MUSCLE DEGENERATION AND CONNECTIVE TISSUE HYPERPLASIA

When guinea-pigs in which a fibula had been fractured and which had been kept for long periods (up to 4 months) on diets deficient in vitamin C were killed, it was frequently noticed that one or both posterior limbs were swollen in the shank region, with tightly drawn skin, a change which did not occur in dietetically normal controls. On dissection, the flesh had a curious gelatinous feel and it was difficult to separate the muscles from one another; often they seemed to have been replaced by a tissue of a different character.

### *Structure of the altered tissue*

The altered tissue was studied in Exps. 3–6. Transverse sections of the swollen shank regions of animals from the partially vitamin C-deficient groups showed that there had been a great increase in the amount of loose connective tissue which, in severe cases, had largely replaced the muscles (Pl. 4, figs. 15, 16). In animals kept for such long periods as 3 months after the operation this tissue had the following character. The cells present were almost all of one kind, and resembled fibroblasts. The intercellular tissue components were a fibrillar framework and the tissue fluid. The fibrillar framework varied a good deal in character. The fibrils were extremely fine, and were often so knitted or matted together as to form the membranous walls of a honeycomb system, in the multitudinous, and of course communicating, cavities of which was the tissue fluid (Pl. 5, fig. 23). The picture was reminiscent of an argyrophil reticulum rather than of a mature areolar connective tissue with its interlacing collagen fibres as its dominant feature. The fibrils stained black with Wilder's silver method, except for the very finest, which we did not succeed in staining at all but made visible by mounting in 'Euparal'. The fibrils took only a weak blue colour with Azan and did not stain with van Gieson. As well as this architecture of extremely fine fibrils, collagen fibres were often also present, and might be a prominent feature in the histological picture.

Besides the formation of the hyperplastic connective tissue replacing the muscles, the normal connective tissue structures, such as fascia and the fibrous layers of periosteum, tended to break down. Fasciae might become completely unrecognizable. The fibrous layers of periosteum never broke down completely, but areas could be found in which the limiting fibrous membrane between the osteogenic tissue and the surrounding hyperplastic connective tissue had suffered dissolution, leaving no histologically recognizable barrier between them (Pl. 4, fig. 17). A similar fate might befall the interosseous ligament between the tibia and fibula, and even tendons.

When muscles were present in the section, depending in part on whether or not they were removed at dissection, they might be normal. On the other hand, they were often oedematous, the fibres not being packed closely together as in a normal muscle, but having wide spaces between them. The connective tissue framework of the muscle might be increased in quantity, as though the muscle were being invaded by the extra-muscular connective tissue, or its own connective tissue proliferating. The muscle fibres had often shrunk away from their envelopes and had lost their characteristic internal structure, the myofibrils being fused into a homogeneous mass.

In places, groups of more or less isolated muscle fibres might be seen wandering

through the hyperplastic connective tissue by which they were surrounded and separated from one another. Such muscle fibres usually seemed to have retained their structure, showing nuclei and striations, but were very small, often extremely so. We find it very difficult to decide whether they were fibres in process of destruction by atrophy, or regenerating fibres which had failed to grow to anything like their normal final size.

Vascularization of the hyperplastic connective tissue was always poor; vessels were few and often without blood corpuscles, especially the arteries. Arteries were frequently seen to have their lumina partially or completely blocked by thickening and vacuolation of the endothelium (see below). Often, but by no means always, large numbers of red blood corpuscles could be seen lying in the tissue spaces of large areas of the connective tissue.

*Development of hyperplastic connective tissue*

The origin and development of this tissue was studied in Exp. 9, in which sixteen young guinea-pigs on a partially vitamin C-deficient diet were killed after fracture of the fibula at intervals given in Table 1. Transverse serial sections were cut across the operated limbs, above and below the site of fracture, and a few longitudinal series were also made.

There was little or no hyperplasia of the connective tissue in animals killed at 3 days after the fracture, but its formation had begun at 5 days, was more obvious at 8 days, and was large in the animals killed at 11 days and later.

The formation of the new tissue was closely bound up with degenerative changes in the muscle. These were seen in the animals killed at 3, 5 and 8 days after the operation, and in one killed at 11 days. In animals killed later, muscular degeneration was much less apparent, but much muscle had disappeared. These changes resulted in the total destruction, so far as could be seen in transverse sections, of many or all of the muscle fibres in entire muscles. The earliest recognizable departure from the normal was an oedema of the muscles in which the fascicles, and also individual muscle fibres, became widely separated from one another (Pl. 5, fig. 18). So far, the individual muscle fibres appeared normal. This simple oedema was seen especially in the earlier killed animals, but was also to be seen much later; it could be, but was not necessarily, a prelude to the destructive changes next to be described. These began with a loss of nuclei and a disappearance of the fibrillar structure of the muscle fibres (Pl. 5, fig. 19). It was difficult to determine exactly what happened to the nuclei; they were either present and apparently normal, or absent. They did not become pycnotic, for such a change would have been easily recognizable, and we think some of them disappeared by a progressive loss of their staining power. In less injured muscle fibres, the nuclei survived and might later take part in what little regeneration occurred. The loss of fibrillar structure appeared as a fusion of the fibrils, making the fibres appear homogeneous in cross-section. This change was often accompanied by an increase in the diameter of the fibre and by a development of vacuoles; these appeared first as many tiny droplets, giving the cross-section of the fibre a pitted appearance (Pl. 5, fig. 20). Later, the droplets might coalesce and form a large central cavity by which the fibre might be blown out to a very large size. Vacuolation of the fibres was

common but not universal; a great many fibres acquired a 'fluffy' appearance and broke up into irregular fragments. Even in muscles in which most of the fibres were undergoing these changes, a few fibres here and there often seemed to remain normal.

While muscle fibres were degenerating, the muscles were invaded by cells from without. Most of these were rather large cells with much cytoplasm and oval or indented nuclei, of the type of mononuclear wandering cells. Mitotic figures were common among them. They were found in large compact masses, having a pseudo-epithelial appearance, in the peripheral parts of the sections. In life, these groups must have been close beneath the skin. From the masses, it was easy to trace migration of the cells to the muscles, into which they penetrated. Within the muscles, they applied themselves to the surfaces of the degenerating muscle fibres, and gave every appearance of being engaged in their destruction (Pl. 5, fig. 21). Multinucleate giant cells were also present, and similarly appeared to be engaged in attacking the degenerate fibres. At a somewhat later stage the muscle fibres, or many of them, had disappeared, while their endomysial or sarcolemmal envelopes persisted as empty tubes whose walls no longer gave with Azan the blue reaction so brilliantly seen in neighbouring normal muscles. The wandering cells could often be seen in occupation of these tubes in place of the original owners (Pl. 5, fig. 22).

While the destruction of muscle fibres was in progress, there was an increase in the fibroblasts between them. This was brought about partly by multiplication of cells in the original connective tissue of the muscle and partly, we suppose, by transformation of the wandering cells. Up to 8 days after the operation, degenerating muscles infiltrated by wandering cells are a predominant feature in the histological picture, while increase in the connective tissue is not yet striking, but from 11 days onwards such gross degenerative changes are scarce or absent while the striking histological feature is the great masses of young connective tissue cells occupying areas which had evidently been muscular, and in which traces of the envelopes of muscle fibres could often easily be seen. In such regions, the fibroblasts of the new connective tissue seem at first comparatively scarce, but there are mitotic figures among them. They multiply, and as they do so a delicate fibrillar reticulum forms, replacing the old architecture of the destroyed muscle, and this soon becomes unrecognizable.

At the same time, fibroblasts of the intermuscular connective tissues also proliferate and so contribute to the developing mass of new connective tissue.

A second mode of formation of the connective tissue was by proliferation of the fibroblasts between the muscle fibres of oedematous muscles whose fibres did not degenerate as described above but, we believe, were reduced in size and number by an atrophy which ended in the disappearance of many fibres.

Still a third mode of origin of the new tissue was by the organization of exudates, which were commonly found in the intermuscular connective tissue. By the penetration of fibroblasts into such exudates, they were built up into parts of the general connective tissue.

It was impossible to tell how much of the hyperplastic connective tissue formed by each of these modes of origin, because the final result was in each case the same, but the largest contribution was certainly made by the destruction of muscle fibres and their replacement by connective tissue.

The connective tissue, formed as just described, did not, within the limits of the

experiment (extending up to 18 days after the fracture), become identical in structure with the fully formed hyperplastic connective tissue which we have described in material fixed some 3 months after the operation. Large parts of it are much more densely cellular than it later becomes, and the honeycomb-like architecture, seen in the older material, has not yet developed, the fibrillar framework making a much more open net-work.

*Regeneration of muscle fibres*

In the developing hyperplastic connective tissue one could very often see a number of very small muscle fibres, wandering in an apparently undirected manner. Many of these were undoubtedly regenerating muscle fibres, for they showed multinucleate regeneration buds, but we suspect that some were fibres in course of atrophy. The regenerates, even in animals which lived for 3 months or more after the operation, remained very much smaller than normal fibres and were few in number; nothing suggested that any more complete regeneration of muscle fibres occurred, such as was described by Le Gros Clark & Blomfield (1945), and as we found in our dietetically normal animals. If such extensive regeneration had occurred in the dietetically partially deficient animals, the observations described above, of the replacement of large areas of muscle by connective tissue, could not have been made.

*Comparison with dietetically normal animals*

The changes described above in animals on diets partially deficient in vitamin C were also seen in those on fully supplemented diets (Exp. 1, and group 1 of Exps. 3–6), but there was a great quantitative difference.

In the three dietetically normal animals killed on the 1st, 2nd and 3rd days after the fracture, a considerable number of changed muscle fibres were found. These fibres showed loss of nuclei, loss of striations, and loss of myofibrils, giving the fibres a homogeneous appearance. Some fibres suffered fragmentation. The muscles were oedematous and there was an extensive polymorph infiltration. Whether there was markedly less of these changes in the first 3 days than was to be seen in the two partially deficient animals killed at 3 days, it is difficult to say.

In the animal killed at 4 days after the fracture, the polymorph infiltration was much less, there was more than the normal amount of connective tissue and this was very cellular and obviously recently formed. Since regenerating muscle fibres were present in it, it had evidently formed in place of destroyed muscle tissue.

In the legs of the remaining animals killed during the first 16 days after the fracture, muscle fibres undergoing degeneration were rare, but regenerating fibres were very numerous, growing through areas of young connective tissue.

We have little doubt that the amount of muscle tissue which was destroyed after suffering injury at the operation was much less in the dietetically normal animals than in the partially deficient, but no quantitative estimate was possible. It is certainly true that the amount of hyperplastic connective tissue formed in the limbs of the partially deficient animals was very much greater than in those on the normal diet; this difference must evidently reflect either a greater quantity of muscle destroyed in the partially deficient animals, or a greater quantity regenerated in the normals, or most probably both.

In dietetically normal animals killed 3 months or more after the operation, there was little or no connective tissue beyond the normal, and no regeneration in progress; the injured fibres had, apparently, long since been restored to the normal condition. This was, of course, in sharp contrast with the partially deficient animals in which the hyperplastic connective tissue was, at corresponding times, at its fullest development, in which there had been very much less regeneration of muscle fibres, and in which such regenerates as did occur failed to attain anything like the normal size.

### *Aetiology*

It is natural to attribute the oedema, the exudates, and the muscular degenerations, to the injury inflicted by the fracture forceps. This is supported by the following figures.

In Exp. 9, in which we studied the development of the connective tissue hyperplasia, killing sixteen animals at various times after the operation, if the formation of the hyperplasia were a consequence of the operation, it would not be expected to occur until several days after it. This was the case, for it had not appeared in animals killed at 3 days, was beginning in those killed at 5 and 8 days, but was large at and after 11 days.

Again, if the hyperplasia were a result of the operation, it should have occurred in the operated and not in the unoperated limbs. Of the sixteen animals in Exp. 9, thirteen showed hyperplasia in the operated leg, and the remaining three, which were those killed at 3 days after the operation, showed in the muscles of the operated legs the changes which precede the formation of hyperplastic connective tissue. Of the sixteen unoperated legs, thirteen showed no sign of hyperplasia, while some hyperplasia was present in three (19 %). In two of these it was much less than on the operated side. In Exps. 5 and 6, nineteen animals on partially deficient diets suffered the operation on one side. There was formation of hyperplastic connective tissue in at least seven of the nineteen operated legs (37 %). In the same experiments, eight other animals were similarly dieted but not operated (groups 5 and 6). Among the thirty-five legs, made up of two from each of these eight and of the nineteen unoperated legs of the animals mentioned above, connective tissue hyperplasia developed in at least eight (23 %). In Exps. 3 and 4, which included nineteen animals kept on diets partially deficient in vitamin C, some connective tissue hyperplasia occurred in at least eight out of twenty-six operated legs (31 %), and in at least one out of twelve legs which were not operated (8 %). In this experiment the operation was performed 2 weeks before the diet was made partially deficient in vitamin C, and during this period repair was proceeding on a fully supplemented diet; nevertheless, the partial deficiency, late though it began, had the effect just stated.

We conclude that (1) the connective tissue hyperplasia developed more readily in operated than in unoperated legs, but (2) it could develop in legs which had suffered no operative interference.

The hyperplasia never developed in the legs of animals receiving 10 mg. of ascorbic acid daily.

The evidence clearly points to an inability of the musculature of the partially vitamin C-deficient guinea-pigs to regenerate after injury, and probably indicates

a greater susceptibility to injury in these animals, than in those supplied with adequate quantities of the vitamin. Why, then, was there in some cases a connective tissue hyperplasia in unoperated limbs? The only answer we can make to this is to suggest that the muscles, which the vitamin deficiency has made more liable to injury, can be so affected by traumatic agents, which would have much less effect on normal limbs, as to bring about the changes which we described above. Possible occasions of such injury were the animals' kicking during the taking of radiographs, in handling during administration of ascorbic acid, etc.

We have examined the blood vessels in sections through the legs of partially vitamin C-deficient and dietetically normal animals. In the operated legs of partially deficient animals the arteries especially were affected. The changes could be recognized in the animals killed at 3 days, and were seen in full development in those killed at 5 days. At this time, and later, many of the vessels were empty of corpuscles, or nearly so, especially the arteries. Again, chiefly in the arteries, the endothelial cells were swollen by vacuoles developed in the basal parts of the cells, between the nuclei and the internal elastic membrane. The vacuoles cause the cells to project into the lumen of the vessel (Pl. 5, figs. 24, 25). Small arteries may be blocked by projecting, and even desquamated, endothelial cells. Delicate strands seen in the sections crossing the lumen from side to side were difficult to interpret with certainty; they seemed to be the walls of very greatly expanded endothelial vacuoles. Vacuoles might also appear beneath the internal elastic membrane and among the smooth muscle fibres. Occasionally, the vacuolation here became so intense that the muscle fibres were loosened up and separated from one another (Pl. 5, fig. 26); rarely, the disintegration process might go so far as a complete breakdown of the cross-section of the vessel (Pl. 5, fig. 27), which might then be histologically recognizable only by tracing a connexion, through serial sections, with its more normal parts. The veins usually appeared normal, and contained blood, with vacuolate and swollen endothelium. Lymphatics were numerous and often greatly expanded; mitotic figures were often seen in their walls.

Examination of the blood vessels in those legs of guinea-pigs which were killed or died at times ranging up to 3 or 4 months after the operation, and which showed hyperplasia of the connective tissue, also showed that the conditions just described in animals killed during the first 18 days after the operation had persisted. The hyperplastic connective tissue was always nearly avascular, and most of the blood vessels (especially the arteries) in or near it were empty or nearly so, with vacuolate and swollen endothelium. When, on the other hand, there was no development of hyperplastic connective tissue, the blood vessels were normal.

Comparison with the operated legs of dietetically normal animals, and especially with those killed in the first 3 weeks, showed that it would be almost true to say that the vascular changes just described did not occur in these animals. In some specimens, however, especially in those whose legs showed any large accumulation of connective tissue, empty vessels, especially arteries, with vacuolate endothelium, could be found. But this was far less frequent than in the partially deficient animals and, when present, was less severe.

The present experiments do not enable us to decide whether the partial deficiency of vitamin C, in preventing adequate regeneration of injured muscles and causing

their replacement by connective tissues, acted directly, by increasing the sensitivity of muscle fibres to injury and/or by reducing their power of recovery and regeneration, or indirectly, as by causing interference with the vascular supply. Le Gros Clark & Blomfield (1945) found that interruption of the arterial supply to the muscles of otherwise normal animals led to degeneration of the muscle fibres and their temporary replacement by connective tissue; when an adequate collateral blood supply had been established, regeneration re-established the muscle and the excess connective tissue melted away. This suggests that the primary effect, in our partially deficient animals, may have been on the blood vessels. The use of the fracture forceps in the operation inevitably injured the muscles by squeezing them, causing haemorrhages by rupturing capillaries and other vessels. Haemorrhagic areas are very common in the muscles of our partially deficient animals, but the degenerative changes we have described often occurred in their absence. The vascular stagnation resulting from the rupture of many small vessels, and the oedematous condition of the muscles, might be expected to cause such anoxaemia as was presumably responsible for the muscular degeneration in Le Gros Clark & Blomfield's experiments, and might also take the blame for the closely similar changes in our material. However this may be, the blood vessels, or at least the arteries, are apparently more liable to injury in the partially deficient animals than in the dietetically normal controls. Meyer, in scorbutic animals, mentions changes in blood vessels similar to those we have described, but unrelated to any known injury and to hyperplastic connective tissue.

Our guinea-pigs were not supplied with any special supplement of vitamin E, and a number of authors (Einarson & Ringsted, 1938; Evans & Burr, 1928; Evans, Emerson & Telford, 1938; Olcott, 1938; Pappenheimer, 1939, 1943) have described degenerative muscular changes, in various species, when deprived of this vitamin. In some respects the changes described above resemble these descriptions, and the question arises whether the changes in our vitamin C-partially deficient animals could have been due to lack rather of vitamin E than of vitamin C. A number of facts tell against this idea. First, the changes in our material were local, whereas those caused by vitamin E deficiency are widely spread through practically the whole skeletal musculature. Secondly, authors describing the muscles in vitamin E deficiency do not mention the wholesale local replacement of muscles by hyperplastic connective tissue. Thirdly, and most convincingly, ascorbic acid protected our animals against the changes we have described, and would not do so against those of vitamin E deficiency.

## SUMMARY

1. In partial vitamin C deficiency the formation of callus at fractures of the fibula was slower, and the amount of callus formed was at first less than in dietetically normal animals.

2. Whereas in normal animals the callus later consolidated into compact bone by thickening of the trabeculae, in the partially deficient animals this did not occur; the callus might become extremely extensive, covering the whole diaphysis (see the second paper of this series), but always retained a lightly built, trabecular structure.

3. In a large proportion of animals kept on partially vitamin C-deficient diets, the knees became stiff and could be bent only painfully and with difficulty. Evidence is presented indicating that this condition was caused at least in part by local histological changes.

4. The manner of fracturing the fibula caused some injury to muscle, and damaged muscle fibres degenerated. In dietetically normal animals they quickly regenerated. In partially vitamin C-deficient animals, much more muscle degenerated than in dietetically normal animals, and the muscular tissue so lost was replaced by large masses of hyperplastic connective tissue and did not regenerate. A large part of the limb musculature might thus disappear. The process of degeneration of the muscle fibres, and the development of the hyperplastic connective tissue, are described.

5. The degeneration of muscles and their replacement by hyperplastic connective tissue occurred more readily in operated legs (in which the muscles were certainly damaged) than in unoperated, but it was also seen in unoperated limbs, though less frequently. It is suggested that the muscles of partially vitamin C-deficient animals are more liable to injury than those of normal animals, and can be damaged by traumatic agents which do not affect those of normals. Possible occasions of such injury were the animal's kicking when being anaesthetized before radiographs were taken.

6. The hyperplastic connective tissue was always avascular or nearly so. Examination of the blood vessels, during the period of muscle degeneration, showed abnormalities especially in the arteries, and these are described. The vessels were found to be in the same condition in the hyperplastic connective tissue of animals which lived for months after the operation. The evidence does not permit us to say whether the replacement of muscles by connective tissue reflected a direct effect of the partial deficiency of vitamin C on the muscle fibres and their ability to regenerate, or an indirect effect through the blood vessels; a certain resemblance of the histological picture to that described by Le Gros Clark & Blomfield, after experimental interruption of the arterial supply, suggests the latter.

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## EXPLANATION OF PLATES

**List of abbreviations used in plates:** *cal.* callus; *cart.* cartilage; *fib.* fibular; *fract.* position of fracture; *n.b.* new bone; *peri.* periosteum; *tib.* tibia.

### PLATE 1

Fig. 1. Normal animal. Stages in the repair of the fractured fibula; from Exp. 6, group 1.

Fig. 2. Partially vitamin C-deficient animal. Stages in the repair of the fractured fibula; from Exp. 6, group 4.

Fig. 3. Normal animal. Stages in the repair of the fractured fibula; from Exp. 5, group 1.

Fig. 4. Partially vitamin C-deficient animal. Stages in the repair of the fractured fibula; from Exp. 5, group 3.

### PLATE 2

Fig. 5. Normal animal. Fibula, 9th day after fracture, showing cartilage (lower right) in the developing callus. Giemsa. ×35. From Exp. 1.

Fig. 6. Normal animal. Fibula, 9th day after fracture. No callus at fracture, but new bone on fibula at some distance from it, and on tibia. Tibia and its marrow on left. Haematoxylin and eosin. ×17. From Exp. 1.

Fig. 7. Normal animal. Fibula, 14th day after fracture, showing callus. Azan. ×38. From Exp. 1.

Fig. 8. Normal animal. Fibula, 16th day after fracture. Haematoxylin and eosin. ×25. From Exp. 1.

### PLATE 3

Fig. 9. Normal animal. Fibula, 101st day after fracture. Bracket indicates consolidated callus. Haematoxylin and eosin. ×24. From Exp. 6, group 1.

Fig. 10. Partially vitamin C-deficient animal. Fibula, 14th day after operation, 40th day of experiment. Extensive resorption and no callus at site of fracture; new subperiosteal bone forming on both stumps. Azan. ×27. From Exp. 9.

Fig. 11. Partially vitamin C-deficient animal. Fibula, 18th day after fracture and 45th day of experiment. Azan. ×58. From Exp. 5, group 4.

Fig. 12. Partially vitamin C-deficient animal. Fibula and part of tibia, 61st day after fracture and 85th day of experiment. Haematoxylin and eosin. ×12. From Exp. 6, group 4.

Fig. 13. Partially vitamin C-deficient animal. Fibula and part of tibia, 93rd day after operation, 120th day of experiment. Haematoxylin and eosin. ×15. From Exp. 5, group 4.

PLATE 4

Fig. 14. Partially vitamin C-deficient animal. Fibula and part of tibia (right), 81st day after operation, 66th day of experimental diets. Haematoxylin and eosin. ×20. From Exp. 4, group 4.

Fig. 15. Partially vitamin C-deficient animal. Part of transverse section of shank, showing hyperplastic connective tissue, 102nd day after operation and 87th day of experimental diets. Haematoxylin and eosin. ×17. From Exp. 3, group 3.

Fig. 16. From the same specimen as fig. 15, showing hyperplastic connective tissue. Azan. ×60.

Fig. 17. Partially vitamin C-deficient animal, 81st day after operation, 67th day after beginning experimental diets. An area of tibial surface where the periosteum has dissolved in the surrounding hyperplastic connective tissue. Haematoxylin and eosin. ×175. From Exp. 4, group 4.

PLATE 5

Fig. 18. Partially vitamin C-deficient animal. An oedematous muscle, and, below, the fibular periosteum, 4th day after operation and 29th day of experiment. Haematoxylin and eosin. ×135. From Exp. 9.

Fig. 19. Partially vitamin C-deficient animal. Early degeneration of muscle fibres which show neither nuclei nor myofibrils, 4th day after operation, 29th day of experiment. Haematoxylin and eosin. ×580. From Exp. 9.

Fig. 20. Partially vitamin-C deficient animal. Degenerating muscle fibres showing 'pitting' by small vacuoles, and fragmentation of fibres, 6th day after operation, 31st day of experiment. Azan. ×600. From Exp. 9.

Fig. 21. Partially vitamin C-deficient animal. Degenerating muscle fibres under attack by mono- and multi-nucleate cells, 6th day after operation and 31st day of experiment. Azan. ×440. From Exp. 9.

Fig. 22. Partially vitamin C-deficient animal. Empty envelopes of muscle fibres occupied by wandering cells, 6th day after operation, 31st day of experiment. Haematoxylin and eosin. ×575. From Exp. 9.

Fig. 23. Partially vitamin C-deficient animal. The hyperplastic connective tissue, from the same specimen as fig. 15. Azan. ×745. From Exp. 3, group 3.

Fig. 24. Partially vitamin C-deficient animal. An artery with vacuolation of the endothelium, 12th day after operation, 37th day of experiment. Haematoxylin and eosin. ×210. From Exp. 9.

Fig. 25. Partially vitamin C-deficient animal. An artery with vacuolate and thickened endothelium, 4th day after operation, 29th day of experiment. Haematoxylin and eosin. ×210. From Exp. 9.

Fig. 26. Partially vitamin C-deficient animal. An artery showing vacuolation in both endothelium and connective tissue of the wall, 19th day after operation, 44th day of experiment. Haematoxylin and eosin. ×210. From Exp. 9.

Fig. 27. Partially vitamin C-deficient animal. Remains of a degenerate artery, only recognizable by tracing through serial sections, 15th day after operation, 40th day of experiment. Haematoxylin and eosin. ×210. From Exp. 9.

Fig. 1.

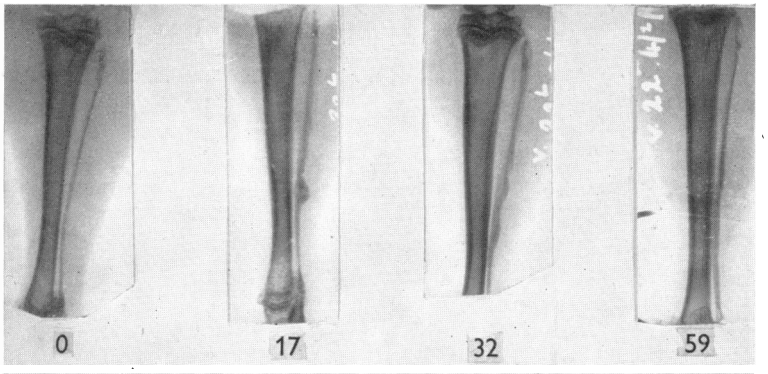
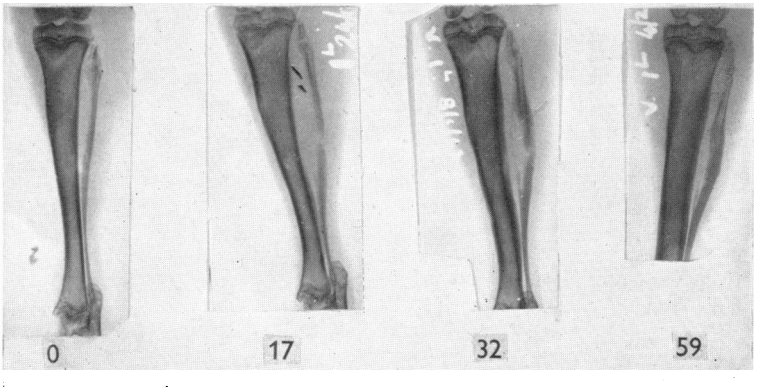



Fig. 2.

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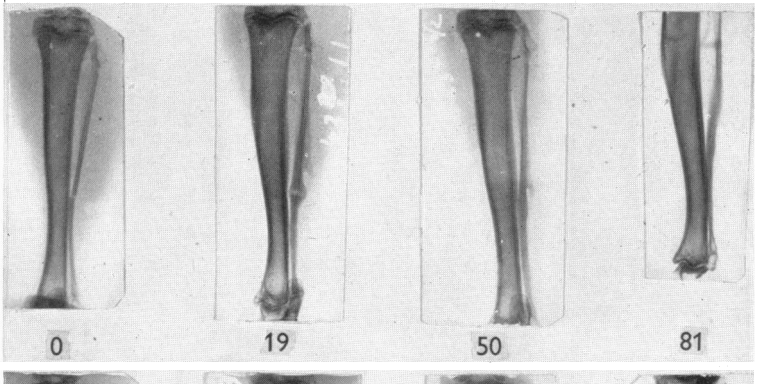


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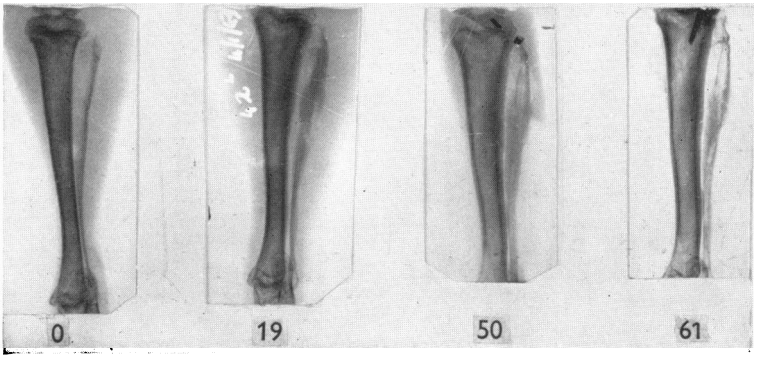


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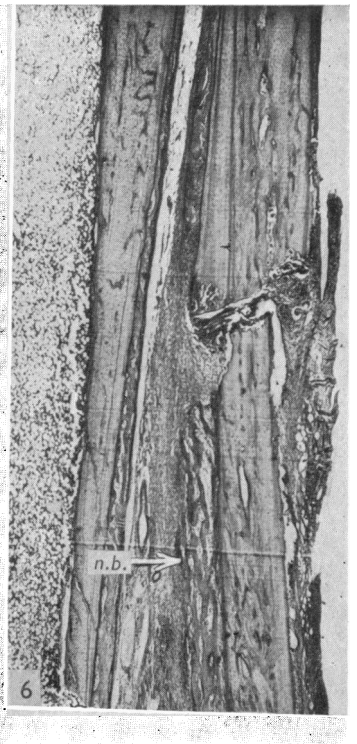
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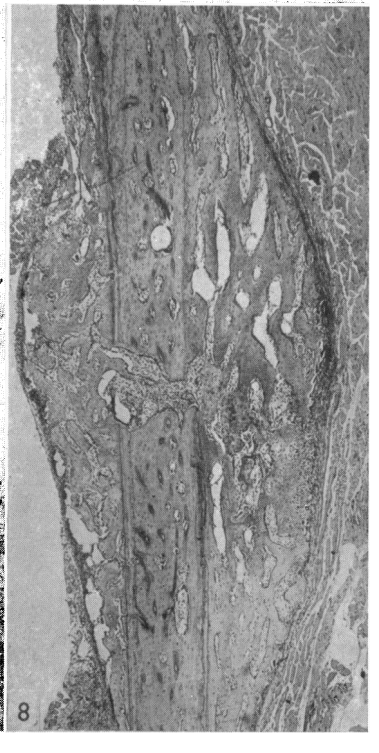
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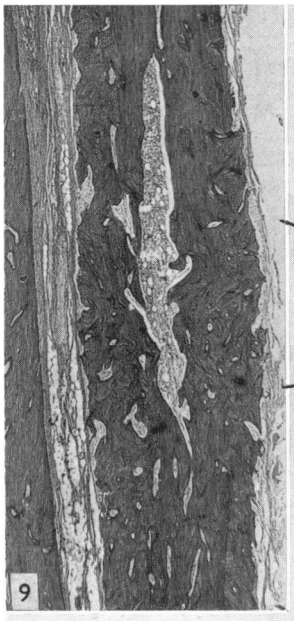
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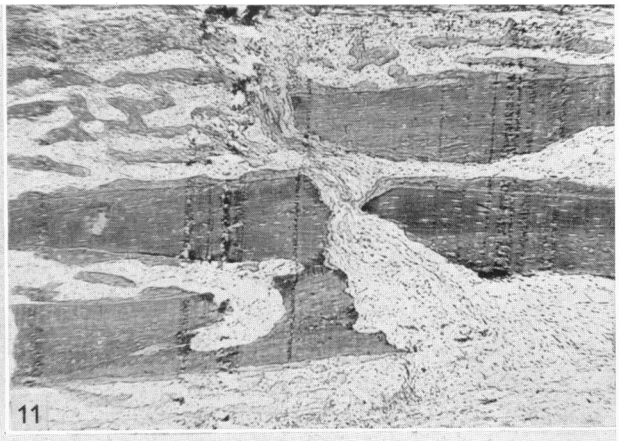
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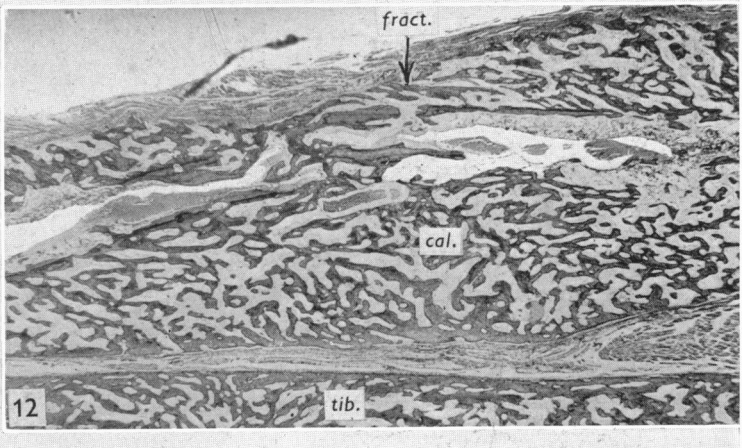
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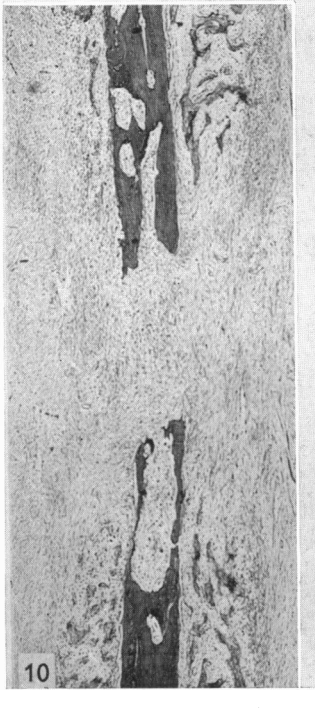
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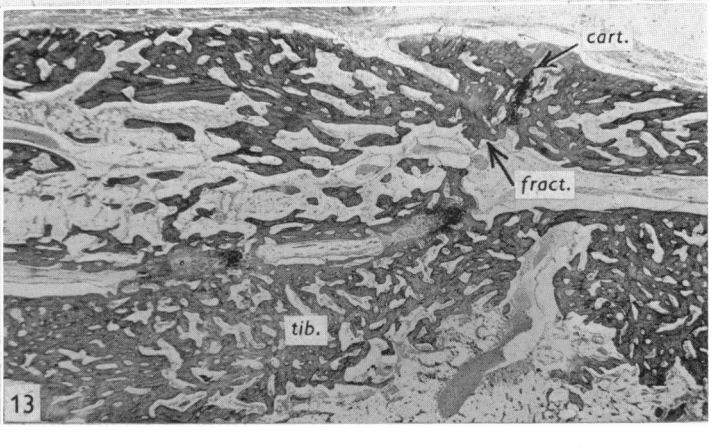
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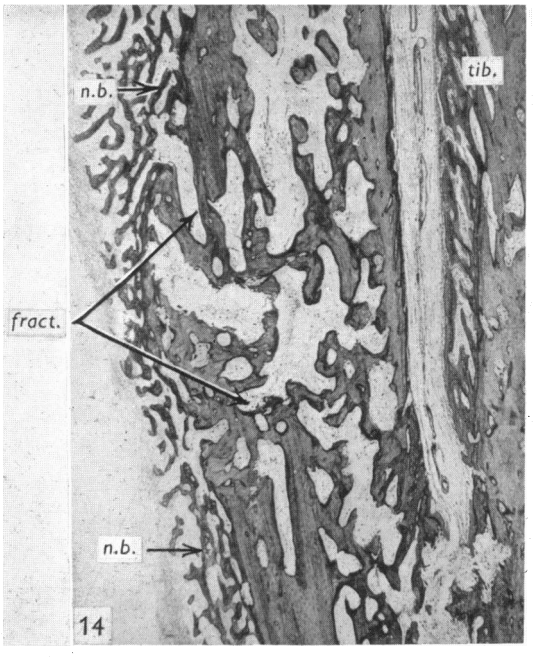
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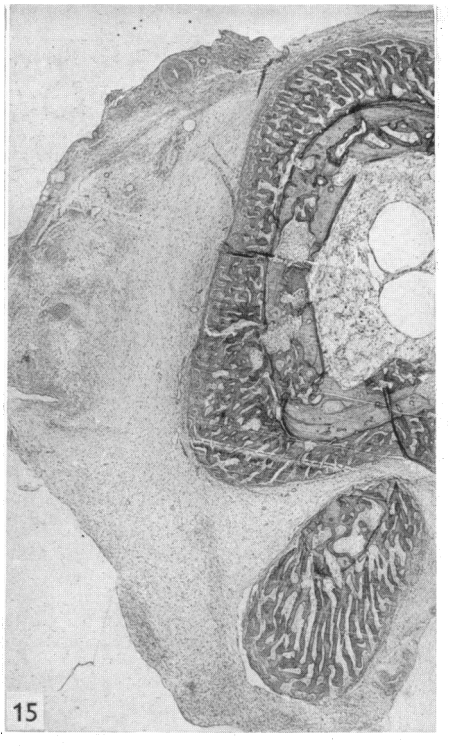
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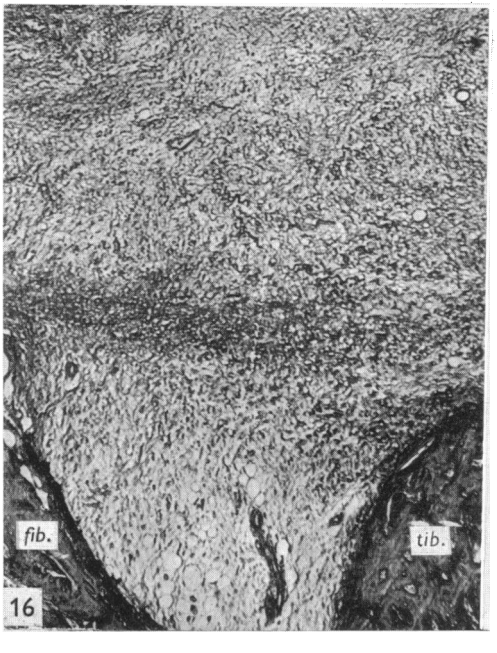
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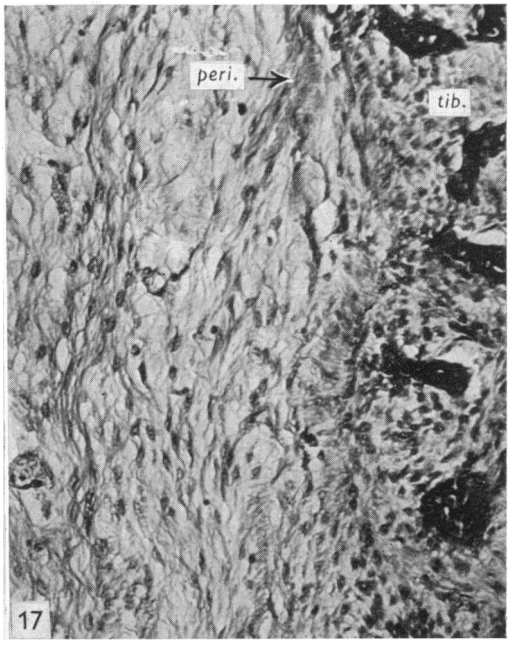
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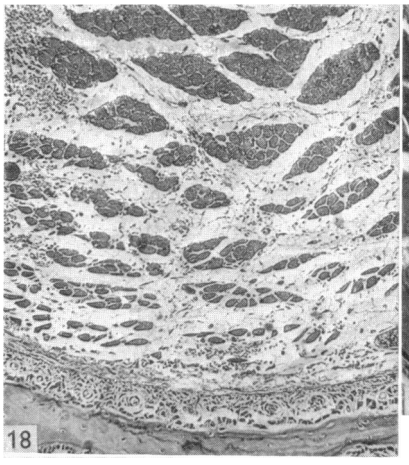
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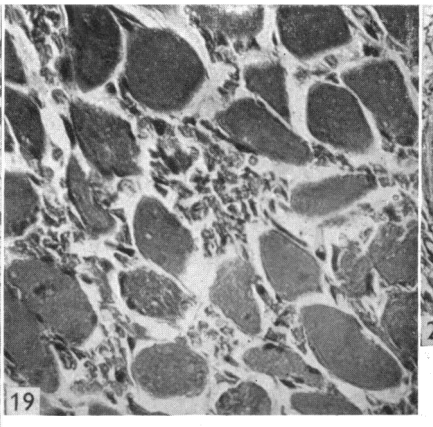
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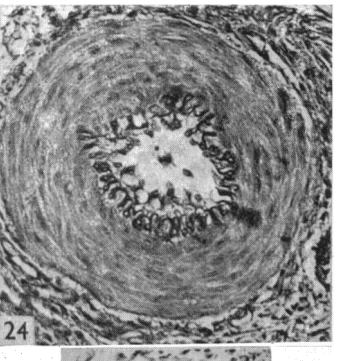
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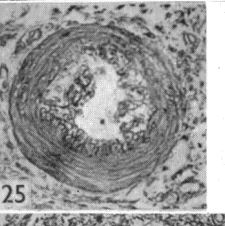
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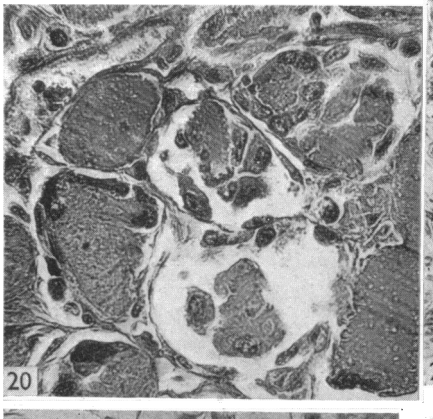
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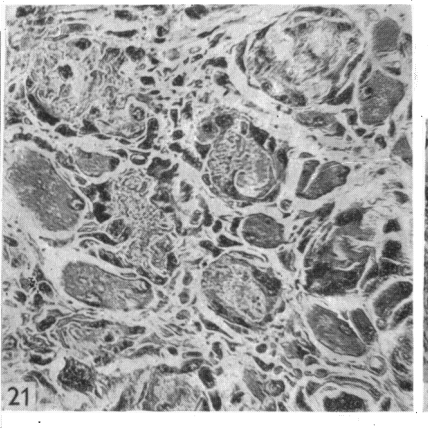
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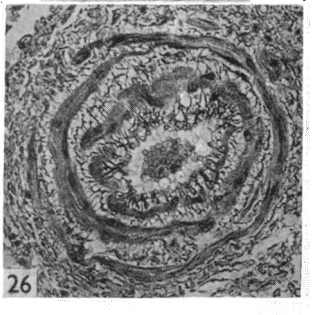
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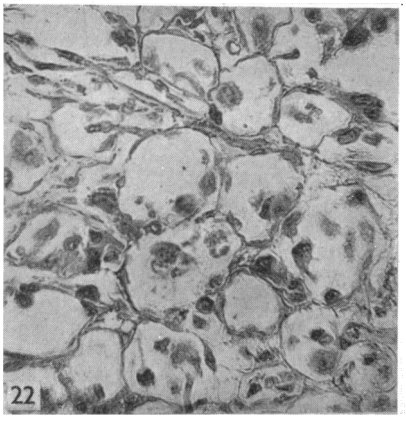
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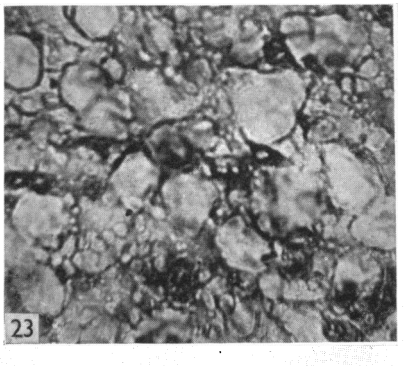
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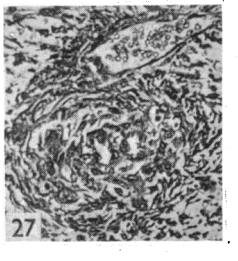
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